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**Applicants: Graham P. Allaway et al.**

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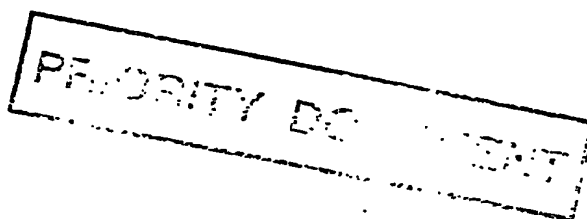
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D. SIEBER

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Page 2 de l'attestation

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Bezeichnung der Erfindung  
Title of the invention  
Titre de l'invention

CC-chemokines receptor and nucleic acid molecule encoding said receptor

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

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Remarques

5

10 CC-CHEMOKINES RECEPTOR AND NUCLEIC ACID MOLECULE ENCODING  
SAID RECEPTOR.

Field of the present invention.

15 The present invention concerns a new receptor which is stimulated by the MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES chemokines.

The present invention concerns also the nucleic acid molecule encoding said receptor, the vector comprising said nucleic acid molecule, cells transformed by said vector, antibodies directed against said receptor, nucleic acid probes directed against said nucleic acid molecule, pharmaceutical compositions comprising said products and non-human transgenic animals expressing the receptor according to the invention or the nucleic acid molecule encoding said  
20 receptor.  
25

The invention further provides a method for determining ligand binding, detecting expression, screening for drugs binding specifically to said receptor and treatments involving the receptor according to the invention.

30

Technological background and state of the art.

Chemotactic cytokines, or chemokines, are small signalling proteins that can be divided in two subfamilies (CC- and CXC-chemokines) depending on the relative position of the first two conserved cysteines. Interleukin 8 (IL-8) is the most studied of these proteins, but a large number of chemokines (Regulated on Activation Normal T-cell Expressed and Secreted (RANTES), Monocyte Chemoattractant Protein 1 (MCP-1), Monocyte Chemoattractant Protein 2 (MCP-2), Monocyte Chemoattractant Protein 3 (MCP-3), Growth-Related gene product  $\alpha$  (GRO $\alpha$ ), Growth-Related gene product  $\beta$  (GRO $\beta$ ), Growth-Related gene product  $\gamma$  (GRO $\gamma$ ), Macrophage Inflammatory Protein 1 $\alpha$  (MIP-1 $\alpha$ ) and  $\beta$ , etc.) has now been described (Baggiolini et al., 1994). Chemokines play fundamental roles in the physiology of acute and chronic inflammatory processes as well as in the pathological dysregulations of these processes, by attracting and stimulating specific subsets of leucocytes (Oppenheim et al., 1991). RANTES for example is a chemoattractant for monocytes, memory T-cells and eosinophils, and induces the release of histamine by basophils. MCP-1, released by smooth muscle cells in arteriosclerotic lesions, is considered as the factor (or one of the factors) responsible for macrophage attraction and, therefore, for the progressive aggravation of the lesions (Baggiolini et al., 1994).

MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES chemokines have recently been described as major HIV-suppressive factors produced by CD8<sup>+</sup> T-cells (Cocchi et al. (1995)). CC-chemokines are also involved in the regulation of human myeloid progenitor cell proliferation (Broxmeyer et al. (1990), Broxmeyer et al. (1993)).

Recent studies have demonstrated that the actions of CC- and CXC-chemokines are mediated by subfamilies of G protein-coupled receptors. To date, despite the numerous functions attributed to chemokines and the increasing number of biologically active ligands, only six functional receptors have been identified in human. Two receptors for interleukin-8 (IL-8) have been described [Holmes et al., 1991; Murphy & Tiffany, 1991]. One (IL-8RA) binds IL-8 specifically, while the other (IL-8RB) binds IL-8 and other CXC-chemokines, like GRO. Among receptors binding CC-chemokines, a receptor, designated CC-chemokine receptor 1 (CC-CKR1), binds both RANTES and MIP-1 $\alpha$  (Neote et al., 1993), and the CC-chemokine receptor 2 (CC-CKR2) binds MCP-1 and MCP-3 (Charo et al., 1994; Yamagami et al., 1994; Franci et al., 1995). Two additional CC-chemokine receptors were cloned recently : the CC-chemokine receptor 3 (CC-CKR3) was found to be activated by RANTES, MIP-1 $\alpha$  and MIP-1 $\beta$  (Combadiere et al., 1995) ; the CC-chemokine receptor 4 (CC-CKR4) responds to MIP-1, RANTES and MCP-1 (Power et al., 1995). In addition to these six functional receptors, a number of orphan receptors have been cloned from human and other species, that are structurally related to either CC- or CXC-chemokine receptors. These include the human BLR1 (Dobner et al., 1992), EBI1 (Birkenbach et al., 1993), LCR1 (Jazin et al., 1993), the mouse MIP-1 RL1 and MIP-1 RL2 (Gao & Murphy, 1995) and the bovine PPR1 (Matsuoka et al., 1993). Their respective ligand(s) and function(s) are unknown at present.

#### Summary of the invention.

This invention provides a new CC-chemokine receptor which is stimulated by the MIP-1 $\beta$  chemokine at a concentration < 10 nM.

The stimulation of the CC-chemokine receptor according to the invention by the MIP-1 $\beta$  chemokine is the biological activity (acidification rate) measured by a microphysiometer as described below.

5 This means that the receptor according to the invention, in presence of this agonist at this concentration or below, presents a functional response (biological activity measured by the acidification rate); while no other known CC-chemokine receptor presents said functional response when it  
10 is stimulated by said agonist at the same concentration.

Advantageously, the receptor according to the invention is also stimulated by the MIP-1 $\alpha$  and RANTES chemokines.

In addition, said receptor is not stimulated by the  
15 MCP-1, MCP-2, MCP-3, IL-8 and GRO $\alpha$  chemokines.

According to a preferred embodiment of the present invention, the receptor is a human receptor.

Said receptor has an amino acid sequence having more than 70% homology with the amino acid sequence shown in  
20 figure 1. Preferably, the amino acid sequence of the purified receptor according to the invention has at least the amino acid sequence shown in figure 1 or a portion thereof.

A portion of an amino sequence means a peptide or a protein having the same binding properties as the whole  
25 receptor according to the invention above-described.

The present invention is also related to a nucleic acid molecule, such as a DNA molecule or a RNA molecule, encoding the receptor according to the invention.

Preferably, said DNA molecule is a cDNA molecule  
30 or a genomic DNA molecule.

Preferably, said nucleic acid molecule has more than 70% homology to the DNA sequence shown in figure 1.

Preferably, the nucleic acid molecule according to the invention is at least the DNA sequence shown in figure 1 or a portion thereof. "A portion of a nucleic acid sequence" means a nucleic acid sequence encoding at least a portion of amino acid sequence as above-described.

The present invention is also related to a vector comprising the nucleic acid molecule according to the invention. Preferably, said vector is adapted for expression in a cell and comprises the regulatory elements necessary for expressing the amino acid molecule in said cell operatively linked to the nucleic acid sequence according to the invention as to permit expression thereof.

Preferably, said cell is chosen among the group consisting of bacterial cells, yeast cells, insect cells or mammalian cells. The vector according to the invention is a plasmid, preferably a pcDNA3 plasmid, or a virus, preferably a baculovirus, an adenovirus or a semliki forest virus.

The present invention concerns also the cell, preferably a mammalian cell, such as a CHO-K1 or a HEK293 cell, transformed by the vector according to the invention. Advantageously, said cell is non neuronal in origin and is chosen among the group consisting of CHO-K1, HEK293, BHK21, COS-7 cells.

The present invention also concerns the cell (preferably a mammalian cell such as a CHO-K1 cell) transformed by the vector according to the invention and by another vector encoding a protein enhancing the functional response in said cell. Advantageously, said protein is the G $\alpha$ 15 or G $\alpha$ 16 (G protein,  $\alpha$  subunit).

The present invention is also related to a nucleic acid probe comprising the nucleic acid molecule according to the invention of at least 15 nucleotides capable of



specifically hybridizing with a unique sequence including with the sequence of the nucleic acid molecule encoding the receptor according to the invention. Said nucleic acid probe may be a DNA or a RNA.

5           The invention concerns also an antisense oligonucleotide having a sequence capable of specifically hybridizing to an mRNA molecule encoding the receptor according to the invention so as to prevent translation of said mRNA molecule or an antisense oligonucleotide having a  
10           sequence capable of specifically hybridizing to the cDNA molecule encoding the receptor according to the invention.

          Said antisense oligonucleotide may comprise chemical analogs of nucleotide or substances which inactivate mRNA, or be included in an RNA molecule endowed with ribozyme  
15           activity.

          Another aspect of the present invention concerns a ligand other than a chemokine (preferably an antibody) capable of binding to a receptor according to the invention and an anti-ligand (preferably also an antibody) capable of  
20           competitively inhibiting the binding of said antibody to the receptor according to the invention.

          Preferably, said antibody is a monoclonal antibody.

          The present invention concerns also the monoclonal antibody directed to an epitope of the receptor according to  
25           the invention and present on the surface of a cell expressing said receptor.

          The invention concerns also the pharmaceutical composition comprising an effective amount of oligonucleotide according to the invention, effective to decrease the  
30           activity of said receptor by passing through a cell membrane and binding specifically with mRNA encoding the receptor according to the invention in the cell so as to prevent it

translation. The pharmaceutical composition comprises also a pharmaceutically acceptable carrier capable of passing through said cell membrane.

Preferably, in said pharmaceutical composition, the  
5 oligonucleotide is coupled to a substance, such as a ribozyme, which inactivates mRNA.

Preferably, the pharmaceutically acceptable carrier comprises a structure which binds to a receptor on a cell capable of being taken up by cell after binding to the  
10 structure. The structure of the pharmaceutically acceptable carrier in said pharmaceutical composition is capable of binding to a receptor which is specific for a selected cell type.

Preferably, said pharmaceutical composition  
15 comprises an amount of the antibody according to the invention effective to block the binding of a ligand to the receptor according to the invention and a pharmaceutically acceptable carrier.

The present invention concerns also a transgenic  
20 non human mammal overexpressing (or expressing ectopically) the nucleic acid molecule encoding the receptor according to the invention.

The present invention also concerns a transgenic non human mammal comprising an homologous recombination  
25 knockout of the native receptor according to the invention.

According to a preferred embodiment of the invention, the transgenic non human mammal whose genome comprises antisense nucleic acid complementary to the nucleic acid according to the invention is so placed as to be  
30 transcribed into antisense mRNA which is complementary to the mRNA encoding the receptor according to the invention and which hybridizes to mRNA encoding said receptor, thereby

reducing its translation. Preferably, the transgenic non human mammal according to the invention comprises a nucleic acid molecule encoding the receptor according to the invention and comprises additionally an inducible promoter  
5 or a tissue specific regulatory element.

Preferably, the transgenic non human mammal is a mouse.

The invention relates to a method for determining whether a ligand can be specifically bound to the receptor  
10 according to the invention, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said receptor with the ligand under conditions permitting binding of ligand to such receptor and detecting the presence of any such ligand bound specifically  
15 to said receptor, thereby determining whether the ligand binds specifically to said receptor.

The invention relates to a method for determining whether a ligand can specifically bind to a receptor according to the invention, which comprises preparing a cell  
20 extract from cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the ligand with the membrane fraction under conditions permitting binding of the ligand to such receptor and detecting the  
25 presence of any ligand bound to said receptor, thereby determining whether the compound is capable of specifically binding to said receptor. Preferably, said method is used when the ligand is not previously known.

The invention relates to a method for determining  
30 whether a ligand is an agonist of the receptor according to the invention, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding

said receptor with the ligand under conditions permitting the activation of a functional receptor response from the cell and detecting by means of a bio-assay, such as a modification in a second messenger concentration (preferably calcium ions or inositol phosphates such as  $IP_3$ ) or a modification in the cellular metabolism (preferably determined by the acidification rate of the culture medium), an increase in the receptor activity, thereby determining whether the ligand is a receptor agonist.

10           The invention relates to a method for determining whether a ligand is an agonist of the receptor according to the invention, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane  
15 fraction from the cell extract, contacting the membrane fraction with the ligand under conditions permitting the activation of a functional receptor response and detecting by means of a bio-assay, such as a modification in the production of a second messenger (preferably inositol  
20 phosphates such as  $IP_3$ ), an increase in the receptor activity, thereby determining whether the ligand is a receptor agonist.

          The present invention relates to a method for determining whether a ligand is an antagonist of the receptor  
25 according to the invention, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said receptor with the ligand in the presence of a known receptor agonist, under conditions permitting the activation of a functional receptor response  
30 and detecting by means of a bio-assay, such as a modification in second messenger concentration (preferably calcium ions or inositol phosphates such as  $IP_3$ ) or a modification in the

cellular metabolism (preferably determined by the acidification rate of the culture medium), a decrease in the receptor activity, thereby determining whether the ligand is a receptor antagonist.

5       The present invention relates to a method for determining whether a ligand is an antagonist of the receptor according to the invention, which comprises preparing a cell extract from cells transfected with an expressing the nucleic acid molecule encoding said receptor, isolating a membrane  
10 - fraction from the cells extract, contacting the membrane fraction with the ligand in the presence of a known receptor agonist, under conditions permitting the activation of a functional receptor response and detecting by means of a bio-assay, such as a modification in the production of a second  
15 messenger, a decrease in the receptor activity, thereby determining whether the ligand is a receptor antagonist.

Preferably, the second messenger assay comprises measurement of calcium ions or inositol phosphates such as  $IP_3$ .

20       Preferably, the cell used in said method is a mammalian cell non neuronal in origin, such as CHO-K1, HEK293, BHK21, COS-7 cells.

The present invention also concerns the cell (preferably a mammalian cell such as a CHO-K1 cell)  
25 transformed by the vector according to the invention and by another vector encoding a protein enhancing the functional response in said cell. Advantageously, said protein is the G $\alpha$ 15 or G $\alpha$ 16 (G protein,  $\alpha$  subunit).

In said method, the ligand is not previously known.

30       The invention is also related to the ligand isolated and detected by any of the preceding methods.

The present invention concerns also the pharmaceutical composition which comprises an effective amount of an agonist or an antagonist of the receptor according to the invention, effective to reduce the activity  
5 of said receptor and a pharmaceutically acceptable carrier.

For instance, said agonist or antagonist may be used in a pharmaceutical composition in the treatment of inflammatory diseases, including rheumatoid arthritis, glomerulonephritis, asthma, idiopathic pulmonary fibrosis and  
10 psoriasis, viral infections including Human Immunodeficiency Viruses 1 and 2 (HIV-1 and 2), cancer including leukaemia, atherosclerosis and/or auto-immune disorders, and the method according to the invention may be advantageously used in the detection of improved drugs which are used in the treatment  
15 of said diseases.

Therefore, the previously described methods may be used for the screening of drugs to identify drugs which specifically bind to the receptor according to the invention.

The invention is also related to the drugs isolated  
20 and detected by any of these methods.

The present invention concerns also a pharmaceutical composition comprising said drugs and a pharmaceutically acceptable carrier.

The invention is also related to a method of  
25 detecting expression of a receptor according to the invention by detecting the presence of mRNA coding for a receptor, which comprises obtaining total RNA or total mRNA from the cell and contacting the RNA or mRNA so obtained with the nucleic acid probe according to the invention under  
30 hybridizing conditions and detecting the presence of mRNA hybridized to the probe, thereby detecting the expression of the receptor by the cell.

Said hybridization conditions are stringent conditions.

The present invention concerns also the use of the pharmaceutical composition according to the invention for the treatment and/or prevention of inflammatory diseases, including rheumatoid arthritis, glomerulonephritis, asthma, idiopathic pulmonary fibrosis and psoriasis, viral infections including Human Immunodeficiency Viruses 1 and 2 (HIV-1 and 2), cancer including leukaemia, atherosclerosis and/or autoimmune disorders.

The present invention concerns also a method for diagnosing a predisposition to a disorder associated with the activity of the receptor according to the invention. Said method comprises :

- a) obtaining nucleic acid molecules of subjects suffering from said disorder;
- b) performing a restriction digest of said nucleic acid molecules with a panel of restriction enzymes;
- c) electrophoretically separating the resulting nucleic acid fragments on a sized gel;
- d) contacting the resulting gel with a nucleic acid probe capable of specifically hybridizing to said nucleic acid molecule and labelled with a detectable marker;
- e) detecting labelled bands which have hybridized to the said nucleic acid molecule labelled with a detectable marker to create a unique band pattern specific to subjects suffering from said disorder;
- f) preparing nucleic acid molecules obtained for diagnosis by step a-e; and
- g) comparing the unique band pattern specific to the nucleic acid molecule of subjects suffering from the disorder from step e and the nucleic acid molecule obtained for

diagnosis from step f to determine whether the patterns are the same or different and to diagnose thereby predisposition to the disorder if the patterns are the same.

5 A last aspect of the present invention concerns a method of preparing the purified receptor according to the invention, which comprises :

- 10 a) constructing a vector adapted for expression in a cell which comprises the regulatory elements necessary for the expression of nucleic acid molecules in the cell operatively linked to nucleic acid molecule encoding said receptor so as to permit expression thereof, wherein the cell is selected from the group consisting of bacterial cells, yeast cells, insect cells and mammalian cells;
- 15 b) inserting the vector of step a in a suitable host cell;
- c) incubating the cell of step b under conditions allowing the expression of the receptor according to the invention;
- d) recovering the receptor so obtained; and
- 20 e) purifying the receptor so recovered, thereby preparing an isolated receptor according to the invention.

Short description of the drawings.

The figure 1 represents the primary structure of a new human chemokine receptor ChemR13.

25 The figure 2 represents the amino acids sequence of the human chemokine receptor ChemR13 according to the invention aligned with that of the human CC-CKR1, CC-CKR2B, CC-CKR3 and CC-CKR4 receptors. Amino acids identical with the ChemR13 sequence are boxed.

30

The figure 3 shows the chromosomal organisation of the human CC-CKR2 and ChemR13 chemokine receptor



genes.

The figure 4 shows the functional expression of the human ChemR13 receptor in a CHO-K1 cell line.

5 The figure 5 represents the distribution of mRNA encoding the ChemR13 receptor in a panel of human cell lines of haematopoietic origin.

Detailed description of the invention.

1. EXPERIMENTALS

Materials

10 Recombinant human chemokines, including MCP-1, MIP-1 $\alpha$ , MIP-1 $\beta$ , RANTES, IL-8 and GRO $\alpha$  were obtained from R & D Systems (London, UK). MCP-2 and MCP-3 were a gift of J. Van Damme, University of Leuven, Belgium. [ $^{125}$ I]MIP-1 $\alpha$  (specific activity, 2200 Ci/mmol) was obtained from Dupont NEN  
15 (Brussels, Belgium). Chemokines obtained from R & D Systems were reported by the supplier as >97 % pure on SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and biologically active on a bioassay specific for each ligand. The lyophilized chemokines were dissolved as a  
20 100  $\mu$ g/ml solution in a sterile phosphate-buffered saline (PBS) and this stock solution was stored at -20° C in aliquots. Chemokines were diluted to the working concentration immediately before use. All cell lines used in  
25 the present study were obtained from the ATCC (Rockville, MD, USA).

Cloning and sequencing

The mouse MOP020 clone was obtained by low stringency polymerase chain reaction, as described previously  
30 (Libert et al., 1989; Parmentier et al., 1989), using genomic DNA as template. A human genomic DNA library (Stratagene, La Jolla, CA) constructed in the lambda DASH vector was screened

at low stringency (Sambrook et al., 1989) with the MOP020 (511 bp) probe. The positive clones were purified to homogeneity and analysed by Southern blotting. The restriction map of the locus was determined and a relevant XbaI fragment of 4,400 bp was subcloned in pBluescript SK+ (Stratagene). Sequencing was performed on both strands after subcloning in M13mp derivatives, using fluorescent primers and an automated DNA sequencer (Applied Biosystem 370A). Sequence handling and data analysis was carried out using the DNASIS/PROSIS software (Hitachi), and the GCG software package (Genetics Computer Group, Wisconsin).

#### Expression in cell lines

The entire coding region was amplified by PCR as a 1056 bp fragment, using primers including respectively the BamHI and XbaI recognition sequences, and cloned after restriction in the corresponding sites of the eukaryotic expression vector pcDNA3 (Invitrogen, San Diego, CA). The resulting construct was verified by sequencing, and transfected in CHO-K1 cells as described (Perret et al., 1990). Two days after transfection, selection for stably transfected cell lines was initiated by the addition of 400 µg/ml G418 (Gibco), and resistant clones were isolated at day 10. CHO-K1 cells were cultured using Ham's F12 medium, as previously described (Perret et al., 1990; Desarnaud et al., 1994). The expression of the ChemR13 receptor in the various cell clones was evaluated by measuring the specific transcript level by Northern blotting, on total RNA prepared from the cells (see below).

### Binding Assays

Stably transfected CHO-K1 cells expressing the ChemR13 receptor were grown to confluence and detached from culture dishes by incubation in phosphate-buffered saline (PBS) supplemented with 1 mM EDTA. Cells were collected by low speed centrifugation and counted in a Neubauer cell. Binding assays were performed in polyethylene minisorp tubes (Nunc) in a final volume of 200  $\mu$ l PBS containing 0.2 % bovine serum albumin (BSA) and  $10^6$  cells, in presence of  $[^{125}\text{I}]\text{-MIP-1}\alpha$ . Non specific binding was determined by addition of 10 nM unlabelled MIP-1 $\alpha$ . The concentration of labelled ligand was 0.4 nM (around 100 000 cpm per tube). The incubation was carried out for 2 hours at 4 °C, and was stopped by the rapid addition of 4 ml ice-cold buffer, and immediate collection of cells by vacuum filtration through GF/B glass fiber filters (Whatmann) pre-soaked in 0.5 % polyethylenimine (Sigma). Filters were washed three times with 4 ml ice-cold buffer and counted in a gamma counter.

### Biological activity

The CHO-K1 cell lines stably transfected with the pcDNA3/ChemR13 construct or wild type CHO-K1 cells (used as controls) were plated onto the membrane of Transwell cell capsules (Molecular Devices), at a density of  $2.5 \times 10^5$  cells/well in Ham's F12 medium. The next day, the capsules were transferred in a microphysiometer (Cytosensor, Molecular Devices), and the cells were allowed to equilibrate for approximately two hours by perfusion of 1 mM phosphate-buffered (pH 7.4) RPMI-1640 medium containing 0.2 % BSA. Cells were then exposed to various chemokines diluted in the same medium, for a 2 min duration. Acidification rates were measured at one minute intervals.

### Northern blotting

Total RNA was isolated from transfected CHO-K1 cell lines, from a panel of human cell lines of haematopoietic origin and from a panel of dog tissues, using the RNeasy kit (Qiagen). RNA samples (10 µg per lane) were denatured in presence of glyoxal (Mc Master & Carmichael, 1977), fractionated on a 1 % agarose gel in a 10 mM phosphate buffer (pH 7.0), and transferred to nylon membranes (Pall Biodyne A, Glen Cove, NY) as described (Thomas, 1980). After baking, the blots were prehybridized for 4h at 42° C in a solution consisting of 50 % formamide, 5x Denhardt solution (1x Denhardt: 0.02 % Ficoll, 0.02 % polyvinylpyrrolidone, 0.02 % BSA), 5x SSPE (1x SSPE: 0.18 M NaCl, 10 mM Na phosphate, 1 mM EDTA pH 8.3), 0.3 % Sodium Dodecyl Sulphate (SDS), 250 µg per ml denaturated DNA from herring testes. DNA probes were ( $\alpha^{32}\text{P}$ )-labelled by random priming (Feinberg & Vogelstein, 1983). Hybridizations were carried out for 12h at 42° C in the same solution containing 10 % (wt/vol) dextran sulphate and the heat denaturated probe. Filters were washed up to 0.1x SSC (1x SSC: 150 mM NaCl, 15 mM Na Citrate pH 7.0), 0.1 % SDS at 60° C and autoradiographed at - 70° C using Amersham  $\beta$ -max films.

## 25 2. RESULTS AND DISCUSSION

### Cloning and structural analysis

The sequence homology characterizing genes encoding G protein-coupled receptors has allowed the cloning by low stringency polymerase chain reaction (PCR) of new members of this gene family (Libert et al., 1989; Parmentier et al., 1989). One of the clones amplified from mouse genomic DNA, named MOP020 presented strong similarities with characterized

chemokine receptors, sharing 80 % identity with the MCP-1 receptor (CC-CKR2) (Charo et al., 1994), 65 % identity with the MIP-1 $\alpha$ /RANTES receptor (CC-CKR1) (Neote et al., 1993), and 51 % identity with IL-8 receptors (Holmes et al., 1991; 5 Murphy & Tiffany, 1991). The clone was used as a probe to screen a human genomic library. A total of 16 lambda phage clones were isolated. It was inferred from the restriction pattern of each clone and from partial sequence data that all clones were belonging to a single contig (see below) in which 10 two different coding sequences were included. One of the coding sequences was identical to the reported cDNA encoding the CC-CKR2 receptor (Charo et al., 1994; Yamagani et al., 1994). A 4.400 pb XbaI fragment of a representative clone containing the second region of hybridization was subcloned 15 in pBluescript SK+. Sequencing revealed a novel gene, tentatively named ChemR13, sharing 84 % identity with the MOP020 probe, suggesting that MOP020 is the mouse ortholog of ChemR13. MOP020 does not correspond to any of the three mouse chemokine receptor genes cloned recently (Gao & Murphy, 20 1995), demonstrating the existence of a fourth murine chemokine receptor.

The sequence of ChemR13 revealed a single open reading frame of 352 codons (fig. 1) encoding a protein of 40,600 Da. The sequence surrounding the proposed initiation 25 codon is in agreement with the consensus as described by Kozak (Kozak, 1989), since the nucleotide in -3 is a purine. The hydropathy profile of the deduced amino acid sequence is consistent with the existence of 7 transmembrane segments. Alignment of the ChemR13 amino acid sequence with that of 30 other functionally characterized human CC-chemokine receptors is represented in figure 1. The highest similarity is found with the CC-CKR2 receptor (Charo et al., 1994) that shares

75.8 % identical residues. There is also 56.3 % identity with the CC-CKR1 receptor (Neote et al., 1993), 58.4 % with the CC-CKR3 (Combadiere et al., 1993), and 49.1% with the CC-CKR4 (Power et al., 1995). ChemR13 represents therefore a new member of the CC-chemokine receptor group (Murphy, 1994). Like the related CC-CKR1 and IL-8 receptors (Holmes et al., 1991; Murphy & Tiffany, 1991; Neote et al., 1993; Gao et al., 1993) the coding region of ChemR13 appears as intronless. From our partial sequencing data, the CC-CKR2 gene is also devoid of intron in the first two thirds of its coding sequence.

Sequence similarities within the chemokine receptor family are higher in the transmembrane-spanning domains, and in intracellular loops. As an example, the identity score between ChemR13 and CC-CKR2 goes up to 92% when considering the transmembrane segments only. Lower similarities are found in the N-terminal extracellular domain, and in the extracellular loops. The N-terminal domain of the IL-8 and CC-CKR2 receptors has been shown to be essential for interaction with the ligand (Hébert et al., 1993; Gong & Clark-Lewis, 1995). The variability of this region among CC-chemokine receptors presumably contributes to the specificity towards the various ligands of the family.

A single potential site for N-linked glycosylation was identified in the third extracellular loop of ChemR13 (figure 1). No glycosylation site was found in the N-terminal domain of the receptor, where most G protein-coupled receptors are glycosylated. The other chemokine receptors CC-CKR1 and CC-CKR2 present such an N-linked glycosylation site in their N-terminal domain (Neote et al., 1993 ; Charo et al., 1994). By contrast, the CC-CKR3 receptor (Combadiere et al., 1995) does not display glycosylation sites neither in

the N-terminus, nor in extracellular loops. The ChemR13 receptor has four cysteines in its extracellular segments, and all four are conserved in the other CC- and CXC-chemokine receptors (figure 2). The cysteines located in the first and  
5 second extracellular loops are present in most G protein-coupled receptors, and are believed to form a disulphide bridge stabilizing the receptor structure (Strader et al., 1994). The two other cysteines, in the N-terminal segment, and in the third extracellular loop could similarly form a  
10 stabilizing bridge specific to the chemokine receptor family (Hébert et al., 1993). The intracellular domains of ChemR13 do not include potential sites for phosphorylation by protein kinase C (PKC) or protein kinase A. PKC sites, involved in heterologous desensitization are frequent in the third  
15 intracellular loop and C-terminus of G protein-coupled receptors. Such sites, present in CC-CKR3 and CC-CKR2 are represented in figure 1. CC-CKR1 is also devoid of PKC sites. In contrast, all CC-chemokine receptors, are rich in serine and threonine residues in the C-terminal domain. These  
20 residues represent potential phosphorylation sites by the family of G protein-coupled receptor kinases, and are probably involved in homologous desensitization (Strader et al., 1994). Five of these S/T residues are perfectly aligned in all five receptors (figure 2).

25

#### Physical linkage of the ChemR13 and CC-CKR2 genes

As stated above, the 16 clones isolated with the MOP020 probe corresponded to a single contig containing the ChemR13 and CC-CKR2 genes. The organisation of this contig  
30 was investigated in order to characterize the physical linkage of the two receptor genes in the human genome. A combination of restriction mapping, Southern blotting,

fragment subcloning and partial sequencing allowed to determine the respective borders and overlaps of all clones. Out of the 16 clones, 9 turned out to be characterized by a specific restriction map, and their organization is depicted in figure 3. Four of these clones (#11, 18, 21, 22) contained the CC-CKR2 gene alone, four clones (# 7, 13, 15, 16) contained the ChemR13 gene alone and one clone (#9) contains part of both coding sequences. The CC-CKR2 and ChemR13 genes are organized in tandem, ChemR13 being located downstream of CC-CKR2. The distance separating CC-CKR2 and ChemR13 open reading frames is 17.5 kb. The chromosomal localization of the tandem is presently unknown. Other chemokine receptors have however been located in the human genome: the CC-CKR1 gene was localized by fluorescence in situ hybridization to the p21 region of human chromosome 3 (Gao et al., 1993). The two IL-8 receptor genes, and their pseudogene have been shown to be clustered on the human 2q34-q35 region (Ahuja et al., 1992). Future studies will demonstrate if CC-chemokine receptor genes do form large clusters in the genome, as do the genes encoding their ligands (Baggiolini et al., 1994).

#### Functional expression and pharmacology of the ChemR13 receptor

Stable CHO-K1 cell lines expressing the ChemR13 receptor were established and were screened on the basis of the level of ChemR13 transcripts as determined by Northern blotting. Three clones were selected and tested for biological responses in a microphysiometer, using various CC- and CXC-chemokines as potential agonists. Wild type CHO-K1 cells were used as control to ensure that the observed responses were specific for the transfected receptor, and did not result from the activation of endogenous receptors. The



microphysiometer allows the real time detection of receptor activation, by measuring the modifications of cell metabolism resulting from the stimulation of intracellular cascades (Owicki & Parce, 1992). Several studies have already  
5 demonstrated the potential of microphysiometry in the field of chemokine receptors. Modifications of metabolic activity in human monocytes, in response CC-chemokines, were monitored using this system (Vaddi & Newton, 1994). Similarly, changes in the acidification rate of THP-1 cells (a human monocytic  
10 cell line) in response to MCP-1 and MCP-3 have been measured (Pleass et al., 1995). The estimation of the EC<sub>50</sub> for both proteins, using this procedure, was in agreement with the values obtained by monitoring the intracellular calcium in other studies (Charo et al., 1994; Franci et al., 1995).

15       Ligands belonging to the CC- and CXC-chemokine classes were tested on the ChemR13 transfected CHO-K1 cells. Whereas MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES were found to be potent activators of the new receptor (figure 4), the CC-chemokines MCP-1, MCP-2 and MCP-3, and the CXC-chemokines GRO $\alpha$  and IL-8  
20 had no effect on the metabolic activity, even at the highest concentrations tested (30 nM). The biological activity of one of the chemokines inducing no response on ChemR13 (IL-8) could be demonstrated on a CHO-K1 cell line transfected with the IL-8A interleukin receptor (Mollereau et al., 1993) : IL-  
25 8 produced a 160 % increase in metabolic activity as determined using the microphysiometer. The biological activity of the MCP-2 and MCP-3 preparations as provided by J. Van Damme have been widely documented (Alam et al., 1994; Sozzani et al., 1994). MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES were tested  
30 on the wild type CHO-K1 cells, at a 30 nM-concentration, and none of them induced a metabolic response. On the ChemR13 transfected CHO-K1 cell line, all three active ligands

(MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES) caused a rapid increase in acidification rate, reaching a maximum by the second or third minute after perfusion of the ligand. The acidification rate returned to basal level within 10 minutes. The timing of the cellular response is similar to that observed for chemokines on their natural receptors in human monocytes (Vaddi & Newton, 1994). When agonists were applied repeatedly to the same cells, the response was strongly reduced as compared to the first stimulation, suggesting the desensitization of the receptor. All measurements were therefore obtained on the first stimulation of each capsule.

The concentration-effect relation was evaluated for the three active ligands in the 0.3 to 30 nM range (figure 3B and C). The rank order of potency was MIP-1 $\alpha$  > MIP-1 $\beta$  = RANTES. At 30 nM concentrations, the effect of MIP-1 $\alpha$  appeared to saturate (at 156 % of baseline level) while MIP-1 $\beta$  and RANTES were still in the ascending phase. Higher concentrations of chemokines could however not be used. The EC50 was estimated around 3 nM for MIP-1 $\alpha$ . The concentrations necessary for obtaining a biological response as determined by using the microphysiometer are in the same range as those measured by intracellular calcium mobilization for the CC-CKR1 (Neote et al., 1993), the CC-CKR2A and B (Charo et al., 1994), and the CC-CKR3 (Combadiere et al., 1995) receptors. The ligand specificity of ChemR13 is similar to that reported for CC-CKR3 (Combadiere et al., 1995). CC-CKR3 was described as the first cloned receptor responding to MIP-1 $\beta$ . However, MIP-1 $\beta$  at 10 nM elicits a significant effect on the ChemR13, while the same concentration is without effect on the CC-CKR3 transfected cells (Combadiere et al., 1995). These data suggest that ChemR13 could be a physiological receptor for MIP-1 $\beta$ .

Binding experiments using [ $^{125}$ I]-human MIP-1 $\alpha$  as ligand did not allow to demonstrate specific binding to ChemR13 expressing CHO-K1 cells, using as much as 0.4 nM radioligand and 1 million transfected cells per tube. Failure to obtain binding data could be attributed to a relatively low affinity of the receptor for MIP-1 $\alpha$  and possibly to an inadequate receptor expression level in our CHO cell line.

#### Northern blotting analysis

Northern blotting performed on a panel of dog tissues did not allow to detect transcripts for ChemR13. Given the role of the chemokine receptor family in mediating chemoattraction and activation of various classes of cells involved in inflammatory and immune responses, the probe was also used to detect specific transcripts in a panel of human cell lines of haematopoietic origin (figure 5). The panel included lymphoblastic (Raji) and T lymphoblastic (Jurkat) cell lines, promyeloblastic (KG-1A) and promyelocytic (HL-60) cell lines, a monocytic (THP-1) cell line, an erythroleukemia (HEL 92.1.7) cell line, a megakaryoblastic (MEG-01) cell line, and a myelogenous leukaemia (K-562) cell line. Human peripheral blood mononuclear cells (PBMC), including mature monocytes and lymphocytes, were also tested. ChemR13 transcripts (4.4 kb) could be detected only in the KG-1A promyeloblastic cell line, but were not found in the promyelocytic cell line HL-60, in PBMC, or in any of the other cell lines tested. These results suggest that the ChemR13 receptor could be expressed in precursors of the granulocytic lineage. CC-chemokines have been reported to stimulate mature granulocytes (McColl et al., 1993; Rot et al., 1992; Kuna et al., 1992; Alam et al., 1992). However, recent data have also demonstrated a role of CC- and CXC-

chemokines in the regulation of mouse and human myeloid progenitor cell proliferation (Broxmeyer et al., 1990; Broxmeyer et al., 1993). The expression of the ChemR13 receptor in normal precursor cells of the granulocyte lineage  
5 will nevertheless have to be confirmed.

REFERENCES

- Ahuja, S.K., Oczelik, T., Milatovich, A., Francke, U., & Murphy, P.M. (1992) *Nature Genetics* 2, 31-36.
- 5 Alam, R., Forsythe, P., Stafford, S., Heinrich, J., Bravo, R., Proost, P., & VanDamme, J. (1994) *J. Immunol.* 153, 3155-3159.
- Alam, R., Forsythe, P.A., Stafford, S., Lett-Brown, M.A., & Grant, J.A. (1992) *J. Exp. Med.* 176, 781-786.
- 10 Baggiolini, M., Dewald, B., & Moser, B. (1994) *Advances in immunology*, Academic press. Ed. Dixon, F.J. 55, 97-179.
- 15 Birkenbach, M., Josefsen, K., Yalamanchili, R., Lenoir, G., & Kieff, E. (1993) *J. Virol.* 67, 2209-2220.
- Broxmeyer, H.E., Sherry, B., Lu, L., Cooper, S., Oh, K., Tekamp-Olson, P., Kwon, B.S., & Cerami, A. (1990) *Blood* 76, 1110-1116.
- 20 Broxmeyer, H.E., Sherry, B., Cooper, S., Lu, L., Maze, R., Beckmann, M.P., Cerami, A., & Ralph, P. (1993) *J. Immunol.* 150, 3448-3458.
- 25 Charo, I.F., Myers, S.J., Herman, A., Franci, C., Connolly, A.J., & Coughlin, S.R. (1994) *Proc. Natl. Acad. Sci.* 91, 2752-2756.
- 30 Cocci, F., DeVico, A.L., Garzino-Demo, A., Arya, S.K., Gallo, R.C., & Lusso, P. (1995) *Science* 270, 1811-1815.

- Combadiere, C., Ahuja, S.K., & Murphy, P.M. (1995) *J. Biol. Chem.* 270, 16491-16494.
- Desarnaud, F., Labbe, O., Eggerickx, D., Vassart, G., &  
5 Parmentier, M. (1994) *Biochem. J.* 299, 367-373.
- Devereux, J., Haeberli, P., & Smithies, O. (1984) *Nucleic Acids Res.* 12, 387-395.
- 10 Dobner, T., Wolf, I., Emrich, T., & Lipp, M. (1992) *Eur. J. Immunol.* 22, 2795-2799.
- Feinberg, A. P., & Vogelstein, B. (1983) *Anal. Biochem.* 132, 6-13.
- 15 Franci, C., Wong, L.M., Van Damme, J., Proost, P., & Charo, I.F. (1995) *J. Immunol.* 154, 6511-6517.
- Gao, J.L., & Murphy, P.M. (1995) *J. Biol. Chem.* 270, 17494-  
20 17501.
- Gao, J.L., Kuhns, D.B., Tiffany, H.L., McDermott, D., Li, X., Francke, U., & Murphy, P.M. (1993) *J. Exp. Med.* 177, 1421-1427.
- 25 Gong, J.H., & Clark-Lewis, I. (1995) *J. Exp. Med.* 181, 631-640.
- Hébert, C.A., Chuntharapai, A., Smith, M., Colby, T., Kim,  
30 J., & Horuk, R. (1993) *J. Biol. Chem.* 268, 18549-18533.

Holmes, W.E., Lee, J., Kuang, V.J., Rice, G.C., & Wood, W.I.  
(1991) *Science* 253, 1278-1283.

- 5 Jazin, E.E., Yoo, H., Blomqvist, A.G., Yee, F., Weng, G., Walker, M.W., Salon, J., Larhammar, D., & Washlestedt, C.  
(1993) *Regul. Peptides* 47, 247-258.

Kozak, M. (1989) *J. Cell. Biol.* 108, 229-241.

10

Kuna, P., Reddigari, S.R., Schall, T.J., Rucinski, D., Viksman, M.Y., & Kaplan, A.P. (1992) *J. Immunol.* 149, 636-642.

- 15 Libert, F., Parmentier, M., Lefort, A., Dinsart, C., Van Sande, J., Maenhaut, C., Simons, M. J., Dumont, J. E., & Vassart, G. (1989) *Science* 244, 569-572.

- 20 Matsuoka, I., Mori, T., Aoki, J., Sato, T., & Kurihara, K.  
(1993) *Biochem. Biophys. Res. Commun.* 194, 504-511.

Mc Master, G. K., & Carmichael, G. G. (1977) *Proc. Natl. Acad. Sci. USA* 74, 4835-4838.

- 25 McColl, S.R., Hachicha, M., Levasseur, S., Neote, K., & Schall, T.J. (1993) *J. Immunol.* 150, 4550-4560.

Mollereau, C., Muscatelli, F., Mattei, M.G., Vassart, G., & Parmentier, M. (1993) *Genomics* 16, 248-251.

30

Murphy, P.M. (1994) *Annu. Rev. Immunol.* 12, 593-633.

- Murphy, P.M., & Tiffany, H.L. (1991) *Science* 253, 1280-1283.
- Neote, K., DiGregorio, D., Mak, J.Y., Horuk, R., & Schall, T.J. (1993) *Cell* 72, 415-425.
- 5 Oppenheim, J.J., Zachariae, C.O., Mukaida, N., & Matsushima, K. (1991) *Ann. Rev. Immunol.* 9, 617-648.
- Owicki, J.C., & Parce, J.W. (1992) *Biosensors Bioelectronics*  
10 7, 255-272.
- Parmentier, M., Libert, F., Maenhaut, C., Lefort, A., Gerard, C., Perret, J., Van Sande, J., Dumont, J. E., & Vassart, G. (1989) *Science* 246, 1620-1622.
- 15 Perret, J., Ludgate, M., Libert, F., Gérard, C., Dumont, J. E., Vassart, G., & Parmentier, M. (1990) *Biochem. Biophys. Res. Commun.* 17, 1044-1050.
- 20 Pleass, R.D., Blythe, T.C., Moore, U.M., Roach, A.G., & Williams, R.J. (1995) *Fourth International Chemokine Symposium, Bath (UK), 27-30 June, P47.*
- Power, C.A., Meyer, A., Nemeth, K., Bacon, K.B., Hoogewerf, A.J., Proudfoot, A.E.I., & Wells T.N.C. (1995) *J. Biol. Chem.* 270, 19495-19500.
- 25 Rot, A., Krieger, M., Brunner, T., Bischoff, S.C., Schall, T.J., & Dahinden, C.A. (1992) *J. Exp. Med.* 176, 1489-1495.



Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

- 5 Sozzani, S., Zhou, D., Locati, M., Rieppi, M., Proost, P.,  
Magazin, M., Vita, N., VanDamme, J., & Mantovani, A. (1994)  
*J. Immunol.* 152, 3615-3622.
- Strader, C.D., Fong, T.M., Tota, M.R., & Underwood, D. (1994)  
10 *Annu. Rev. Biochem. Ed. Dixon, R.A.F.* 63, 101-132.
- Thomas, P. S. (1980). *Proc. Natl. Acad. Sci. USA* 77, 5201-  
5205.
- 15 Vaddi, K., & Newton, R.C. (1994) *The FASEB Journal* 8, A502.
- Yamagami, S., Tokuda, Y., Ishii, K., Tanaka, H., & Endo, N.  
(1994) *Biochem. Biophys. Res. Commun.* 202, 1156-1162.

CLAIMS.

1. CC-chemokine receptor which is stimulated by the MIP-1 $\beta$  chemokine at a concentration  $\leq$  10 nM.
2. Receptor according to the claim 1, which is  
5 stimulated by the MIP-1 $\alpha$  and RANTES chemokines.
3. Receptor according to the claim 1 or 2, which is not stimulated by the MCP-1, MCP-2, MCP-3, IL-8 and GRO $\alpha$  chemokines.
4. Receptor according to any of the preceding  
10 claims, being a human receptor.
5. Receptor according to any of the preceding claims, which has an amino acid sequence having more than 70% homology with the amino acid sequence shown in Figure 1.
6. Receptor according to any of the preceding  
15 claims, which has at least the amino acid sequence shown in Figure 1 or a portion thereof.
7. Nucleic acid molecule encoding the receptor according to any of the preceding claims.
8. Nucleic acid molecule according to claim 7,  
20 wherein the nucleic acid molecule is DNA or RNA molecule.
9. DNA molecule according to claim 8, which is a cDNA molecule or a genomic DNA molecule.
10. Nucleic acid molecule according to any of the claims 7 to 9, having more than 70% homology to the DNA  
25 sequence shown in Figure 1.
11. DNA molecule according to claim 10, which has at least the DNA sequence as shown in figure 1 or a portion thereof.
12. Vector comprising the nucleic acid molecule  
30 according to any of the claims 9 to 11.
13. Vector of claim 12, adapted for expression in a cell, which comprises the regulatory elements necessary for

expression of the nucleic acid molecule in said cell operatively linked to the nucleic acid molecule according to any of the claims 7 to 11 as to permit expression thereof.

14. Vector of claim 13, wherein the cell is chosen  
5 among the group consisting of bacterial cells, yeast cells, insect cells or mammalian cells.

15. Vector according to any of the claims 12 to 14, wherein the vector is a plasmid or a virus, preferably a baculovirus, an adenovirus or a semliki forest virus.

10 16. Vector of claim 15, wherein the plasmid is the pcDNA3 plasmid.

17. Cell comprising the vector according to any of the claims 12 to 16.

X 18. Cell according to claim 17, characterized in  
15 that it is transformed also by another vector encoding a protein enhancing the functional response in said cell, preferably said protein being the Gal5 or the Gal6 protein.

19. Cell of claim 17 or 18, wherein the cell is a mammalian cell, preferably non neuronal in origin.

20 20. Cell of claim 19, wherein the cell is chosen among the group consisting of CHO-K1, HEK293, BHK21, COS-7 cells.

21. Nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically  
25 hybridizing with an unique sequence included within the nucleic acid molecule according to any of the claims 7 to 11.

22. Nucleic acid probe of claim 21, wherein the nucleic acid is DNA or RNA.

23. Antisense oligonucleotide having a sequence  
30 capable of specifically hybridizing to an mRNA molecule of claim 8, so as to prevent translation of the mRNA molecule.

24. Antisense oligonucleotide having a sequence capable of specifically hybridizing to the DNA molecule of claim 9.

25. Antisense oligonucleotide according to claim  
5 23 or 24, comprising chemical analogs of nucleotides.

26. Ligand capable of binding to a receptor according to any of the claims 1 to 6.

27. Anti-ligand capable of competitively inhibiting the binding of the ligand according to claim 26 to the  
10 receptor according to any of the claims 1 to 6.

28. Ligand according to claim 26, which is an antibody.

29. Anti-ligand according to the claim 27, which is an antibody.

15 30. Antibody according to the claim 28 or 29, which is a monoclonal antibody.

31. Monoclonal antibody according to claim 30, directed to an epitope of the receptor according to any of the claims 1 to 6, present on the surface of a cell  
20 expressing said receptor.

32. Pharmaceutical composition comprising an amount of the oligonucleotide according to claim 23, effective to decrease activity of the receptor according to any of the claims 1 to 6 by passing through a cell membrane and binding  
25 specifically with mRNA encoding said receptor in the cell so as to prevent its translation, and a pharmaceutically acceptable carrier capable of passing through a cell membrane.

33. Pharmaceutical composition of claim 32, wherein  
30 the oligonucleotide is coupled to a substance which inactivates mRNA.

34. Pharmaceutical composition of claim 33, wherein the substance which inactivates mRNA is a ribozyme.

35. Pharmaceutical composition according to any of the claims 32 to 34, wherein the pharmaceutically acceptable  
5 carrier comprises a structure which binds to a receptor on a cell capable of being taken up by cell after binding to the structure.

36. Pharmaceutical composition of claim 35, wherein the structure of the pharmaceutically acceptable carrier is  
10 capable of binding to a receptor which is specific for a selected cell type.

37. Pharmaceutical composition which comprises an effective amount of the anti-ligand of claim 27, effective to block binding of a ligand to the receptor according to any  
15 of the claims 1 to 6 and a pharmaceutically acceptable carrier.

38. Transgenic non human mammal expressing the nucleic acid molecule according to any of the claims 7 to 11.

39. Transgenic non human mammal comprising an  
20 homologous recombination knockout of the native receptor according to any of the claims 1 to 6.

40. Transgenic non human mammal whose genome comprises antisense nucleic acid complementary to the nucleic acid molecule according to any of the claims 7 to 11 so  
25 placed as to be transcribed into antisense mRNA which is complementary to the mRNA of claim 8 and which hybridizes to said mRNA thereby reducing its translation.

41. Transgenic non human mammal according to any of the claims 38 to 40, wherein the nucleic acid according  
30 to any of the claims 7 to 11 additionally comprises an inducible promoter.

42. Transgenic non human mammal according to any of the claims 38 to 41, wherein the nucleic acid according to claim 7 to 11 additionally comprises tissue specific regulatory elements.

5           43. Transgenic non human mammal according to any of the claims 38 to 42, which is a mouse.

          44. Method for determining whether a ligand can specifically bind to a receptor according to any of the claims 1 to 6, which comprises contacting a cell transfected  
10 with a vector expressing the nucleic acid molecule encoding said receptor with the ligand under conditions permitting binding of ligand to such receptor and detecting the presence of any such ligand bound specifically to said receptor, thereby determining whether the ligand binds specifically to  
15 said receptor.

          45. Method for determining whether a ligand can specifically bind to the receptor according to any of the claims 1 to 6, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid  
20 molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the ligand with the membrane fraction under conditions permitting binding of the ligand to such receptor and detecting the presence of any ligand bound to said receptor, thereby determining whether  
25 the compound is capable of specifically binding to said receptor.

          46. Method for determining whether a ligand is an agonist of the receptor according to any of the claims 1 to 6, which comprises contacting a cell transfected with a  
30 vector expressing the nucleic acid molecule encoding said receptor with the ligand under conditions permitting the activation of a functional receptor response from the cell

and detecting by means of a bio-assay, such as a second messenger response, an increase in the receptor activity, thereby determining whether the ligand is a receptor agonist.

47. Method for determining whether a ligand is an  
5 agonist of the receptor according to any of the claims 1 to 6, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the membrane  
10 fraction with the ligand under conditions permitting the activation of a functional receptor response and detecting by means of a bio-assay, such as a second messenger response, an increase in the receptor activity, thereby determining whether the ligand is a receptor agonist.

15 48. Method for determining whether a ligand is an antagonist of the receptor according to any of the claims 1 to 6, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said receptor with the ligand in the presence of a known receptor  
20 agonist, under conditions permitting the activation of a functional receptor response and detecting by means of a bio-assay, such as a second messenger response, a decrease in the receptor activity, thereby determining whether the ligand is a receptor antagonist.

25 49. Method for determining whether a ligand is an antagonist of the receptor according to any of the claims 1 to 6, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane  
30 fraction from the cell extract, contacting the membrane fraction with the ligand in the presence of a known receptor agonist, under conditions permitting the activation of a

functional receptor response and detecting by means of a bio-assay, such as a second messenger response, a decrease in the receptor activity, thereby determining whether the ligand is a receptor antagonist.

5           50. A method according to any of the claims 44 to 49, wherein the second messenger assay comprises measurement of calcium ions ( $\text{Ca}^{2+}$ ), inositol phosphates (such as  $\text{IP}_3$ ), diacylglycerol (DAG) or cAMP.

10           51. Method according to any of the preceding claims 44 to 50, wherein the cell is a mammalian cell, preferably non neuronal in origin, and chosen among the group consisting of CHO-K1, HEK293, BHK21 and COS-7 cells.

          52. Method according to any of the preceding claims 44 to 51, wherein the ligand is not previously known.

15           53. Ligand detected by the method according to any of the preceding claims 44 to 52.

          54. Pharmaceutical composition which comprises the ligand according to claim 53 and a pharmaceutically acceptable carrier.

20           55. Method of screening drugs to identify drugs which specifically bind to the receptor according to any of the claims 1 to 6 on the surface of the cell, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said receptor with a plurality  
25 of drugs under conditions permitting binding of said drugs to the receptor, and determining those drugs which specifically bind to the transfected cell, thereby identifying drugs which specifically bind to the receptor.

30           56. Method of screening drugs to identify drugs which specifically bind to the receptor according to any of the claims 1 to 6 on the surface of the cell, which comprises preparing a cell extract from cells transfected with a vector



expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the membrane fraction with a plurality of drugs and determining those drugs which bind to the transfected  
5 cell, thereby identifying drugs which specifically bind to said receptor.

57. Method of screening drugs to identify drugs which act as agonists of the receptor according to any of the claims 1 to 6, which comprises contacting a cell transfected  
10 with a vector expressing the nucleic acid molecule encoding said receptor with a plurality of drugs under conditions permitting the activation of a functional receptor response, and determining those drugs which activates such receptor using a bio-assay, such as a second messenger response,  
15 thereby identifying drugs which act as receptor agonists.

58. Method of screening drugs to identify drugs which act as agonists of the receptor according to any of the claims 1 to 6, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid  
20 molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the membrane fraction with a plurality of drugs under conditions permitting the activation of a functional receptor response, and determining those drugs which activate such receptor  
25 using a bio-assay, such as a second messenger response, thereby identifying drugs which act as receptor agonists.

59. Method of screening drugs to identify drugs which act as antagonists of the receptor according to any of the claims 1 to 6, which comprises contacting a cell  
30 transfected with a vector expressing the nucleic acid molecule encoding said receptor with a plurality of drugs in the presence of a known receptor agonist, under conditions

permitting the activation of a functional receptor response, and determining those drugs which inhibit the activation of the receptor using a bio-assay, such as a second messenger response, thereby identifying drugs which act as receptor antagonists.

60. Method of screening drugs to identify drugs which act as antagonists of the receptor according to any of the claims 1 to 6, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the membrane fraction with a plurality of drugs in presence of a known receptor agonist, under conditions permitting the activation of a functional receptor response, and determining those drugs which inhibit the activation of the receptor using a bio-assay, such as a second messenger response, thereby identifying drugs which act as receptor antagonists.

61. Method according to any of the claims 46 to 49 and 57 to 60, wherein the functional response detected by means of a bioassay is detected and measured by a microphysiometer.

62. Drug detected by any of the methods according to claims 55 to 61.

63. Pharmaceutical composition comprising a drug according to claim 62 and a pharmaceutically acceptable carrier.

64. Method of detecting the expression of the receptor according to any of the claims 1 to 6, by detecting the presence of mRNA coding said receptor, which comprises obtaining total mRNA from the cell and contacting the mRNA so obtained with the nucleic acid probe according to claim 20 under hybridizing conditions, and detecting the presence

of mRNA hybridized to the probe, thereby detecting the expression of the receptor by the cell.

65. Method of detecting the presence of the receptor according to any of the claims 1 to 6 on the surface  
5 of a cell, which comprises contacting the cell with the anti-  
ligand of claim 27 under conditions permitting binding of the  
antibody to the receptor, and detecting the presence of the  
antibody bound to the cell, thereby detecting the presence  
of the receptor on the surface of the cell.

10 66. Method of determining the physiological effects  
of expressing varying levels of the receptor according to any  
of the claims 1 to 6, which comprises producing a transgenic  
non human mammal according to any of the claims 38 to 43  
whose levels of receptor expression are varied by use of an  
15 inducible promoter which regulates the receptor regulation.

67. Method of determining the physiological effects  
of expressing varying levels of the receptor according to any  
of the claims 1 to 6, which comprises producing a panel of  
transgenic non human mammals according to any of the claims  
20 38 to 43, each expressing a different amount of said  
receptor.

68. Method for identifying an antagonist of the  
receptor according to any of the claims 1 to 6 capable of  
alleviating an abnormality in a subject wherein the  
25 abnormality is alleviated by decreasing the activity of the  
receptor, which comprises administering the antagonist to a  
transgenic non human mammal according to any of the claims  
38 to 43 and determining whether the antagonist alleviates  
the physical and behavioural abnormalities displayed by the  
30 transgenic non human mammal as a result of receptor activity,  
thereby identifying the antagonist.

69. Antagonist identified by any of the methods of the claims 64 to 68.

70. Pharmaceutical composition comprising an antagonist according to claim 69 and a pharmaceutically acceptable carrier.

71. Method for identifying an agonist of the receptor according to any of the claims 1 to 6 capable of alleviating an abnormality in a subject wherein the abnormality is alleviated by activation of said receptor, which comprises administering the agonist to a transgenic non human mammal according to any of the claims 38 to 43 and determining whether the antagonist alleviates the physical and behavioural abnormalities displayed by the transgenic non human mammal, the alleviation of the abnormalities indicating the identification of the agonist.

72. Agonist identified by the method of claim 71.

73. Pharmaceutical composition comprising an agonist according to claim 72 and a pharmaceutically acceptable carrier.

74. Method for diagnosing a predisposition to a disorder associated with the activity of a specific allele of the receptor according to any of the claims 1 to 6, which comprises :

- a) obtaining nucleic acid molecules of subjects suffering from said disorder;
- b) performing a restriction digest of said nucleic acid molecules with a panel of restriction enzymes;
- c) electrophoretically separating the resulting nucleic acid fragments on a sized gel;
- d) contacting the resulting gel with a nucleic acid probe capable of specifically hybridizing to said nucleic acid molecule and labelled with a detectable marker;

- e) detecting labelled bands which have hybridized to the said nucleic acid molecule labelled with a detectable marker to create a unique band pattern specific to subjects suffering from said disorder;
- 5 f) preparing nucleic acid molecules obtained for diagnosis by step a-e; and
- g) comparing the unique band pattern specific to the nucleic acid molecule of subjects suffering from the disorder from step e and the nucleic acid molecule obtained for  
10 diagnosis from step f to determine whether the patterns are the same or different and to diagnose thereby predisposition to the disorder if the patterns are the same.

75. Method of preparing the purified receptor  
15 according to any of the claims 1 to 6, which comprises :

- a) constructing a vector adapted for expression in a cell which comprises the regulatory elements necessary for the expression of nucleic acid molecules in the cell  
20 operatively linked to nucleic acid molecule encoding said receptor so as to permit expression thereof, wherein the cell is selected from the group consisting of bacterial cells, yeast cells, insect cells and mammalian cells;
- b) inserting the vector of step a in a suitable host cell;
- c) incubating the cell of step b under conditions allowing  
25 the expression of the receptor according to the invention;
- d) recovering the receptor so obtained; and
- e) purifying the receptor so recovered, thereby preparing an isolated receptor according to the invention.

30 76. Use of the pharmaceutical composition according to any of the claims 32 to 37, 54, 63, 70 and 73, for the preparation of a medicament in the treatment of a disease

chosen from the group consisting of inflammatory diseases,  
including rheumatoid arthritis, glomerulonephritis, asthma,  
idiopathic pulmonary fibrosis and psoriasis, viral infections  
including infections by Human Immunodeficiency Viruses 1 and  
5 2 (HIV-1 and 2), cancer including leukaemia, atherosclerosis  
and/or auto-immune disorders.

ABSTRACTCC-CHEMOKINES RECEPTOR AND NUCLEIC ACID MOLECULE ENCODING  
SAID RECEPTOR.

5

The present invention concerns a new CC-chemokine receptor which is stimulated by the MIP-1 $\beta$  chemokine at a concentration  $\leq$  10 nM.

10 The present invention concerns also the nucleic acid molecule encoding said receptor, the vector comprising said nucleic acid molecule, cells transformed by said vector, antibodies directed against said receptor, nucleic acid probes directed against said nucleic acid molecule, pharmaceutical compositions comprising said products and non-  
15 human transgenic animals expressing the receptor according to the invention or the nucleic acid molecule encoding said receptor.

(Figure 5)

	GAATTCCCCCAACAGAGCCAAGCTCTCCATCTAGTGGACAGGGAAGCTAGCAGCAAACC	59
60	TTCCCTTCACTACAAAACCTTCATTGCTTGGCCAAAAAGAGAGTTAATTCAATGTAGACAT	119
120	CTATGTAGGCAATTA AAAACCTATTGATGTATAAAACAGTTTGCAATTCATGGAGGGCAAC	179
	M E G N	4
180	TAAATACATTCTAGGACTTTATAAAAGATCACTTTTTATTTATGCACAGGGTGAACAAG	239
5	* I H S R T L * K I T F Y L C T G W N K	24
240	ATGGATTATCAAGTGTCAAGTCCAATCTATGACATCAATTATTATACATCGGAGCCCTGC	299
25	M D Y Q V S S P I Y D I N Y Y T S E P C	44
300	CAAAAAATCAATGTGAAGCAAATCGCAGCCCGCCTCCTGCCTCCGCTCTACTCACTGGTG	359
45	Q K I N V K Q I A A R L L P P L Y S L V	64
360	TTCATCTTTGGTTTTGTGGGCAACATGCTGGTCACTCCTCATCCTGATAAACTGCAAAGG	419
65	F I P G F V G N M L V I L I L I N C K R	84
420	CTGAAGAGCATGACTGACATCTACCTGCTCAACCTGGCCATCTCTGACCTGTTTTTCCTT	479
85	L K S M T D I Y L L N L A I S D L F F L	104
480	CTTACTGTCCCTTCTGGGCTCACTATGCTGCCGCCAGTGGGACTTTGGAAATACAATG	539
105	L T V P F W A H Y A A A Q W D P G N T M	124
540	TGTCAACTCTTGACAGGGCTCTATTTTATAGGCTTCTTCTCTGGAATCTTCTTCATCATC	599
125	C Q L L T G L Y F I G F F S G I F F I I	144
600	CTCCTGACAATCGATAGGTACCTGGCTGTGCTCCATGCTGTGTTTGCTTTAAAAGCCAGG	659
145	L L T I D R Y L A V V H A V P A L K A R	164
660	ACGGTCACCTTTGGGGTGGTGACAAGTGTGATCACTTGGGTGGTGGCTGTGTTTGCGTCT	719
165	T V T F G V V T S V I T W V V A V P A S	184
720	CTCCCAGGAATCATCTTTACCAGATCTCAAAAAGAAGGTCTTCATTACACCTGCAGCTCT	779
185	L P G I I F T R S Q K E G L H Y T C S S	204
780	CATTTTCCATACAGTCAGTATCAATTCTGGAAGAATTTCCAGACATTAAAGATAGTCATC	839
205	H F P Y S Q Y Q F W K N F Q T L K I V I	224
840	TTGGGGCTGGTCTCGCGCTGCTTGTCTATGGTCACTGCTACTCGGGAATCTTAAAAACT	899
225	L G L V L P L L V M V I C Y S G I L K T	244
900	CTGCTTCGGTGTGCGAAATGAGAAGAAGAGGCACAGGGCTGTGAGGCTTATCTTCACCATC	959
245	L L R C R N E K K R H R A V R L I F T I	264
960	ATGATTGTTTATTTCTCTTCTGGGCTCCCTACAACATTGTCTTCTCTCTGAACACCTTC	1019
265	M I V Y F L F W A P Y N I V L L L N T F	284
1020	CAGGAATTCTTTGGCCTGAATAATTGCAGTAGCTCTAACAGGTTGGACCAAGCTATGCAG	1079
285	Q E F F G L N N C S S S N R L D Q A M Q	304
1080	GTGACAGAGACTCTTGGGATGACGCACTGCTGCATCAACCCCATCATCTATGCCCTTTGTC	1139
305	V T E T L G M T H C C I N P I I Y A F V	324
1140	GGGAGAAGTTCAGAAACTACCTCTTAGTCTTCTTCCAAAAGCACATTGCCAAACGCTTC	1199
325	G E K F R N Y L L V P F Q K H I A K R F	344
1200	TGCAAATGCTGTTCTATTTTCCAGCAAGAGGCTCCCGAGCGAGCAAGCTCAGTTTACACC	1259
345	C K C C S I F Q Q B A P E R A S S V Y T	364
1260	CGATCCACTGGGGAGCAGGAAATATCTGTGGGCTTGTGACACGGACTCAAGTGGGCTGGT	1319
365	R S T G E Q E I S V G L *	
1320	GACCCAGTCAGAGTTGTGCACATGGCTTAGTTTTATACACAGCCTGGGCTGGGGGTNGG	1379
1380	TTGGNNGAGGTCTTTTTTAAAAGGAAGTTACTGTTATAGAGGGTCTAAGATTTCATCCATT	1439
1440	TATTTGGCATCTGTTTAAAAGTAGATTAGATCCGAATTC	

FIG.1



FIG. 2

hChemR13	1 M YQVSSPI	II	ILINCKRLKSMTDIYLLNLAISDI	83
hCC-CXR2b	MI,STGIRSRIRIN'NESGEEV'TTFFI	I	LLPPI.YSLVIFGFGNMLV	95
hCC-CXR3	MTTSI		LLPPLYSLVIFGFGNMLV	87
hCC-CXR1	MFVETEGTTSYYDDVGL		LLPPLYSLVIFGFGNMLV	87
hCC-CXR4	METPNTTDEDYDTTTEFD		LLPPLYSLVIFGFGNMLV	92
	MNPTDIADTTLDESIYSN	III	LLPPLYSLVIFGFGNMLV	
hChemR13	VPEWAIHYA	IV	VIIA VFA LKARTVTG VVT SVITW	177
hCC-CXR2b	LP LWAHISA		VIIA VFA LKARTVTG VVT SVITW	189
hCC-CXR3	LPFWIIYVRGIINWVF		VIIA VFA LKARTVTG VVT SVITW	182
hCC-CXR1	LPFWITKDKDWEF		VIIA VFA LKARTVTG VVT SVITW	182
hCC-CXR4	LPFWGYVA		VIIA VFA LKARTVTG VVT SVITW	186
hChemR13	YTCS	V	VIIA VFA LKARTVTG VVT SVITW	272
hCC-CXR2b	YTC		VIIA VFA LKARTVTG VVT SVITW	280
hCC-CXR3	YTC		VIIA VFA LKARTVTG VVT SVITW	276
hCC-CXR1	YTC		VIIA VFA LKARTVTG VVT SVITW	276
hCC-CXR4	YTC		VIIA VFA LKARTVTG VVT SVITW	279
hChemR13	SS	VII	VIIA VFA LKARTVTG VVT SVITW	352
hCC-CXR2b	SS		VIIA VFA LKARTVTG VVT SVITW	360
hCC-CXR3	SS		VIIA VFA LKARTVTG VVT SVITW	355
hCC-CXR1	SS		VIIA VFA LKARTVTG VVT SVITW	355
hCC-CXR4	SS		VIIA VFA LKARTVTG VVT SVITW	360

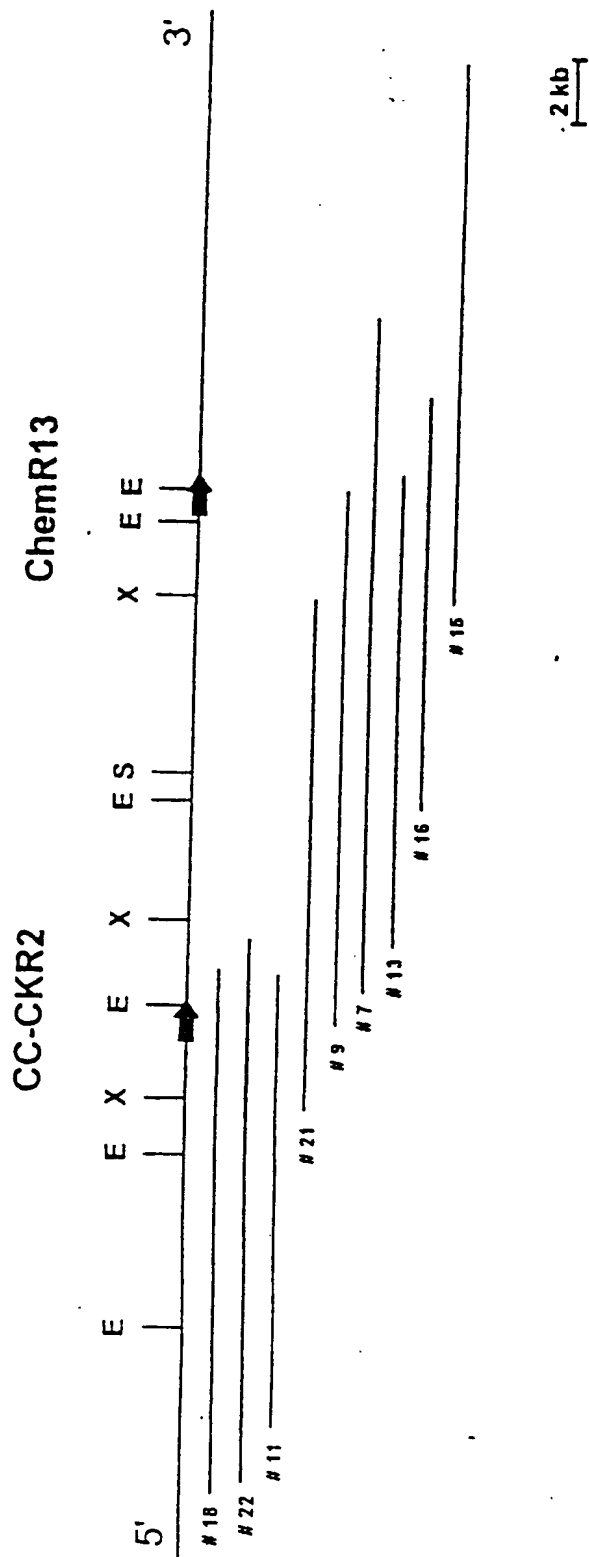


FIG. 3

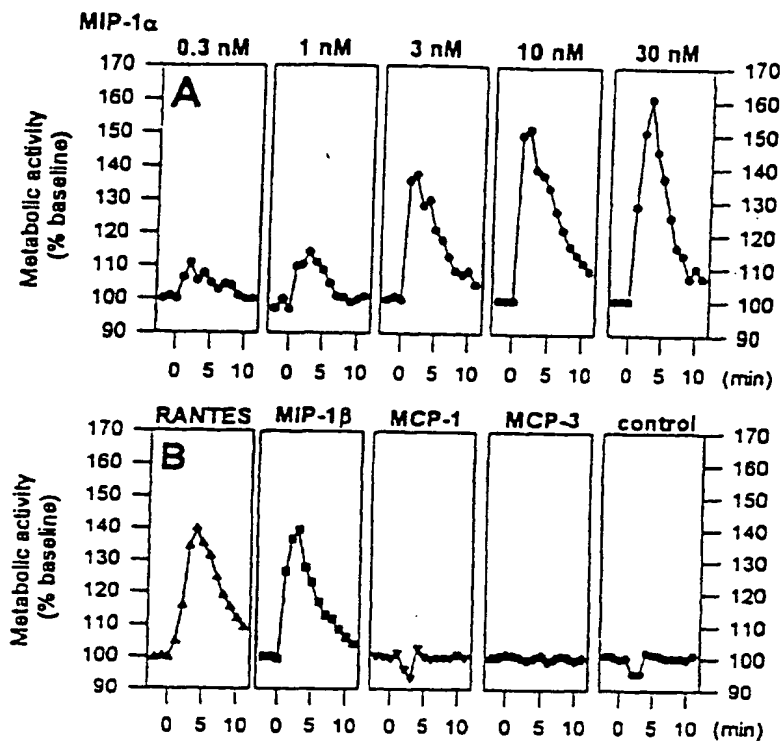


FIG. 4a

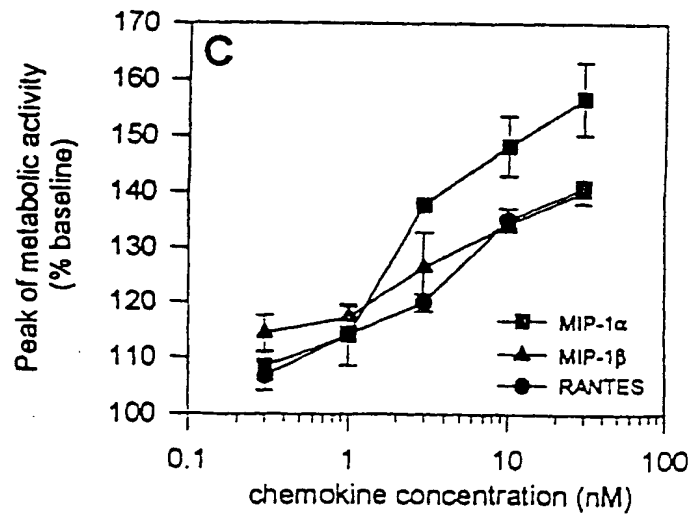


FIG. 4b

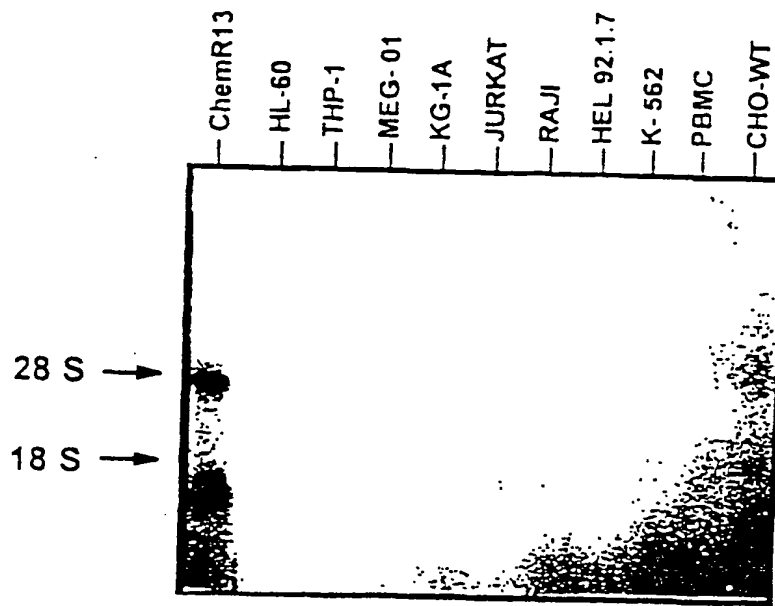


FIG. 5

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